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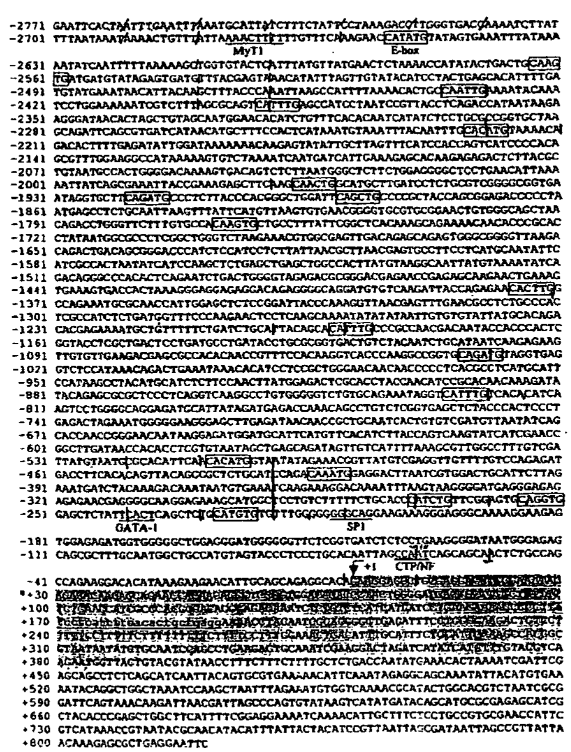

(57) Abstract: The present invention relates to a zebrafish *HuC* promoter with its 5'-flanking region which directs the neuron-specific expression of structural genes, a transgenic animal that shows the neuron-specific expression of GFP (green fluorescence protein) under the regulation of the *HuC* promoter, and a method for screening neurogenesis mutants in zebrafish by use of the transgenic animal. The *HuC* promoter with its 5'-flanking region can be used in the study of the regulatory mechanism responsible for the differentiation of the nervous system. Additionally, the *HuCP-GFP* transgenic zebrafish enables the direct identification of neurogenesis and axonogenesis, as well as being a valuable tool for isolating and analyzing neurogenesis mutants in live zebrafish with ease.

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ZEBRAFISH *HuC* PROMOTER CAPABLE OF DIRECTING NEURON-SPECIFIC EXPRESSION OF STRUCTURAL GENES, TRANSGENIC ANIMAL HAVING *HuC* PROMOTER AND ITS GENERATION, AND METHOD FOR SCREENING NEURONAL MUTANT ANIMALS USING THE TRANSGENIC ANIMAL

FIELD OF THE INVENTION

The present invention relates to a zebrafish *HuC* promoter that drives the neuron-specific expression of structural genes, and a transgenic animal having the *HuC* promoter and its generation. Also, the present invention is concerned with a method for screening neuronal mutants, using the transgenic animal.

BACKGROUND OF THE INVENTION

In gene expression, transcriptional regulation is very important for rapid responses to external signals and establishment of development. Primary spatial and temporal regulation of gene expression is conducted at the transcription level, in which transcription regulatory proteins recognize specific DNA sequence regions near promoters to specifically control the synthesis of mRNA. To express a certain gene in a specific tissue and/or at a specific time, the promoter of the gene and neighboring regions to which

transcription regulatory proteins bind are therefore momentous.

Besides transcription factors, factors that are involved in the regulation of biosynthesis of proteins from gene information include those that are related to the stability of mRNAs produced from genes and that serve to carry mRNAs to the cytosol, particularly, to designated locations within the cytosol. Not only do proteins that play certain roles in the regulation of gene expression have motifs which recognize specific sites of mRNAs, but also expression of their genes are tissue-specific or time-specific according to development stages (Burd and Dreyfuss, 1994).

Belonging to a family of vertebrate neuron-specific genes, *HuC* is known to be highly homologous to the *Drosophila elav*, a vital gene indispensable for the development and maintenance of the nervous system (Good, 1995; Kim et al., 1996). Although much needs to be done to elucidate its functions, vertebrate *HuC* protein was reported to be able to bind AU-rich 3'-untranslated regions (UTRs) of mRNAs for various transcription factors and cytokines and thus believed to play an important role in postmitotic neuronal differentiation and subsequent maintenance of the vertebrate nervous system (Levine et al., 1993; King et al., 1994; Liu et al., 1995; Ma et al., 1996b; Chen and Shyu, 1995).

Essential to the development and maintenance of the nervous system, the *Drosophila elav* protein is the first case of a RNA-binding protein which is expressed specifically in neuronal tissues. *Drosophila elav* was identified on the basis of its RNA-binding motif, which suggests that the elav protein might be related to neuronal RNA metabolism (Robinow et al., 1988).

Studies on elav proteins in the whole developmental process using antibodies have disclosed that the elav protein 1) is expressed during the early stage of neuronal differentiation, 2) appears throughout the central nervous system and peripheral nervous system during the progression of nervous system development, 3) is translocated into nuclei, and 4) is not found in neuroblasts nor glial cells (Robinow et al., 1988, 1991). These results lead to the inference that *elav* functions as a housekeeping gene required for the development and maintenance of neurons.

Due to its requirement in neurons from an early stage of differentiation, *elav* has been used as an early neuronal marker and examination of its expression has helped study cellular, molecular, and genetic interactions that control early neurogenesis in *Drosophila* (Campos et al., 1987; Robinow and White, 1988). *HuC,* a vertebrate homologue of *elav,* has been suggested as a useful tool in the study of early

neurogenesis in zebrafish (Kim et al., 1996) as recent studies have emphasized similarities in the mechanisms that control early neurogenesis in Drosophila and vertebrates, particularly in zebrafish and Xenopus embryos.

In zebrafish, early neurons are distributed in three longitudinal columns of the neural plate. Within these longitudinal columns only a subset of cells express *HuC* and differentiate into neurons. Neurogenin1 (ngn1), a basic helix-loop-helix (bHLH) transcription factor, is limitedly found only in the longitudinal domains where cells have the potential to become neurons, among the distributed columns. That is, the expression of *neurogenin1* (*ngn1*) helps define the longitudinal proneuronal domains (Blader et al., 1997; Kim et al., 1997; Korzh et al., 1998). However, *ngn1* drives the expression of the inhibitory ligand *Delta*A, which interacts with its receptor, Notch, in neighboring cells whose activation, in turn, reduces the expression of *ngn1* in these cells. As a consequence of this inhibitory feedback loop, only a subset of cells manage to maintain high levels of *ngn1* and *DeltaA* expression (Appel and Eisen, 1998; Haddon et al., 1998)). Cells that do these feedback operations begin expressing another *Delta* homologue, *DeltaB* and genes like *MyT1* and *Zcoe2* that facilitate the stable adoption of a neuronal fate (Bellefroid et

al, 1996; Bally-Cuif el al., 1998). These cells also begin to express neuroD, another bHLH transcription factor whose activity leads to expression of early markers of neuronal differentiation like *HuC* (Korzh et al., 1998). Neighboring cells, in which neuronal fate is suppressed by Notch activation, adopt alternate fates, or remain undifferentiated, giving rise to neurons later in development. When the function of the neurogenic genes like *Notch* and *Delta* is suppressed, loss of lateral inhibition leads to the overproduction of *HuC*-expressing cells (Appel and Eisen, 1998).

Zebrafish are now widely used in genetic screening to identify genes responsible for a range of early developmental events. They are particularly well suited to genetic analysis because large numbers of embryos can be easily obtained and raised to maturity within a relatively short period. Furthermore, the embryos are completely transparent during the first day of development (Chitins and Kuwada, 1990, Wilson et al., 1990).

Through large-scale mutagenesis screening, there have been already identified a number of mutants in which the early pattern of neurons is altered, for which the expression of *HuC* was used as an early neuronal marker. In this regard, in order to identify zebrafish mutants in which the distribution of *HuC* mRNA is altered, an approach was used where the

embryos were screened for changes in the distribution of *HuC*-expressing cells by in situ hybridization. The success of this screening demonstrated the value of *HuC* as an early neuronal marker. However, RNA *in situ* hybridization suffers from the disadvantage of making it impossible to directly observe changes in the nervous system of live embryos because the chemicals used for the hybridization kill the embryos. Another problem with the screening method using RNA in situ hybridization is that a complex, time-consuming procedure such as mRNA synthesis, etc. is required. Accordingly, conventional screening methods using in situ hybridization cannot be applied for live embryos owing to their limitations in screening neurogenesis mutants in live embryos and analyzing alterations of neurogenesis therein. Therefore, there remains a need for an improved method that is able to directly identify and analyze alterations in early patterns of neurons of living embryos.

SUMMARY OF THE INVENTION

Leading to the present invention, the intensive and thorough research on the early stages of differentiation of neurons resulted in the finding that 2.8 kb of the 5′-flanking sequence of a zebrafish *HuC* gene is sufficient to restrict GFP (green

fluorescence protein) gene expression to neurons, in which the core promoter spans 251 base pairs and contains a CCAAT box and one SP1 sequence, while no TATA boxes are present near the transcription initiation site. It was also found that a putative MyT1 binding site and at least 17 E-box sequences are necessary to maintain the neuronal specificity of *HuC* expression. Sequential removal of the putative MyT1 binding site and 14 distal E boxes leads to a progressive expansion of GFP expression into muscle cells. Further removal of the three proximal E boxes eliminates neuronal and muscle specificity of GFP expression and leads to ubiquitous expression of GFP in the whole body. Using the *HuC* promoter, a stable zebrafish transgenic line (*HuCP-GFP*) can be established in which GFP is expressed specifically in neurons. By taking advantage of this stable zebrafish transgenic line, neurogenesis mutants in live zebrafish can be visibly identified with ease.

Therefore, it is an object of the present invention to provide a *HuC* promoter that drives the neuron-specific expression of structural genes.

It is another object of the present invention to provide a fused gene construct in which an exogenous GFP gene is expressed under the regulation of the *HuC* promoter.

It is a further object of the present invention

to provide a transgenic animal which harbors the fused gene construct in its genome.

It is still a further object of the present invention to provide a method for generating the transgenic animal.

It is still another object of the present invention to provide a method for screening and analyzing neurogenesis mutants in live zebrafish embryos.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows fluorescence photographs which compare the expression of *HuC* (A) and *Delta*B (B) mRNA in the neuronal plate at the 3-somite stage in dorsal views with anterior to the left. In this figure, ps stands for primary sensory neuron; pin for primary intermediate neuron; and pmn for primary motor neuron.

Fig. 2 is a base sequence showing the structure of the 5′-flanking region, including promoter, of the zebrafish *HuC* gene, in which various symbols or letters are used to denote special functions. The major transcription initiation site is presented as position +1 and marked by an arrow. The shaded letters mark the exon-1 and underlined lowercase letters denote the oligonucleotide sequence corresponding to the antisense oligonucleotide primer used for primer

extension. Bold letters ATG stand for the translation start codon, MyT1, GATA-1 and SP1 sites are underlined. The canonical CBF/Ny-Y binding site (CCAAT-box) is double underlined and E-boxes are boxed.

Fig. 3 is an autoradiogram showing the determination of the transcription initiation site of *HuC* gene by primer extension.

Fig. 4 is a schematic diagram showing the structure of the zebrafish *HuC* promoter in embryos, along with their transient expression patterns of GFP in neurons, muscle cells and other tissues upon the introduction of deletion constructs.

Fig. 5 shows photographs taken of live, 48-hpf zebrafish embryos microinjected with ΔEco under a photo-field, which show the neuronal specificity of gene expression driven by the *HuC* promoter construct visualized through the transiently expressed GFP fluorescence

(A) generated by superimposing a bright-field image on a fluorescence image throughout the whole body with the dorsal part at the top and the anterior to the left;

(B) detected in the nervous system including the telencephalic cluster, retinal ganglion cells, medial longitudinal fasciculus, and dorsal longitudinal fasciculus;

(C) detected in the trigeminal ganglion neuron

and Rohon-Beard neurons (arrows); and

(D) and (E) detected in the peripheral process of Rohon-Beard axons (arrow) and dorsal longitudinal fasciculus of spinal cord. Throughout the photographs, the dorsal part and the anterior part are located at the top and the left, respectively, and the abbreviation dlf stands for dorsal longitudinal fasciculus; ey for eye; mlf for medial longitudinal fasciculus; rb for Rohon-Beard neurons; rg for retinal ganglion; tc for telencephalic cluster; and tg for trigeminal ganglion.

Fig. 6 shows photographs taken of live, 48-hpf zebrafish embryos, which exhibit GFP expression patterns for functional analysis of deletion constructs,

(A) when ΔHind construct was microinjected into one-cell stage embryos;

(B) when ΔBst construct was microinjected into one-cell stage embryos;

(C) when ΔSac construct was microinjected into one-cell stage embryos; and

(D) when ΔSac construct was microinjected into four-cell stage embryos.

Fig. 7 shows photographs taken of live transgenic zebrafish embryos, which exhibit GFP fluorescence detected in

(A) the neurons of a 24-hpf heterozygotic

transgenic embryo in a lateral view;

(B) the neurons of a 24-hpf homozygotic transgenic embryo in a lateral view;

(C) the cranial ganglia highlighted by asterisks and in ventral motor roots of the boxed area marked by arrows; and

(D) the spinal cord of a 60-hpf transgenic zebrafish embryo in a lateral view. In these figures, rb stands for Rohon-Beard cells, co for commissural neurons, and mo for primary motorneurons.

Fig. 8 shows photographs taken of the homozygotic transgenic zebrafish embryos, which exhibit temporal and spatial expression patterns of the *HuCP-GFP* fused gene construct,

(A) detected by whole mount in situ hybridization using a synthetic antisense RNA probe for GFP mRNA transcripts in a dorsal view of an 11-hpf embryo;

(B) detected by whole mount in situ hybridization using a synthetic antisense RNA probe for *HuC* mRNA transcripts in a dorsal view of an11-hpf embryo;

(C) through the expression of acetylated α-tubulin detected by whole mount immunostaining in a lateral view; and

(D) detected in the telencephalic cluster (tc), anterior commissure (ac), epiphysial cluster (ec), posterior commissure (pc), tract of posterior commissure (tpc), postoptic commissure (poc), and

tract of the postoptic commissure (tpoc) of a 24 hpf embryo by anti-GFP antibodies in a lateral view;

(E) detected in the olfactory placodes in an anterior view;

(F) detected in medial longitudinal fasciculus (MLF) and its nucleus (nMLF) in a dorsal view;

(G) detected in the trigeminal ganglion (tg) and rhombomeres (v) in the hindbrain in a dorsal view of the hindbrain.

Fig. 9 is a photograph showing living mib mutant transgenic embryos visualized by GFP fluorescence, in which the neurogenic phenotype in 2-day-old *HuCP-GFP*$^{+/-}$ /mib$^{-/-}$ zebrafish embryo seen by GFP fluorescence with a Leica MZFLIII fluorescence stereomicroscope (right) is compared with a heterozygotic wild-type *HuCP-GFP*$^{+/-}$ transgenic embryo (left).

DETAILED DESCRIPTION OF THE INVENTION

In one aspect of the present invention, there is provided a *HuC* promoter governing the regulation of which structural genes are specifically expressed in neurons.

HuC, which is expressed from *HuC*, belongs to the Hu family of proteins which have RNA-recognition motifs and are a type of RNA-binding proteins which take part in RNA metabolism, such as rRNA production,

translation initiation, structural RNA production, and transportation of RNA to the cytoplasm. Of the human Hu proteins identified thus far, HuD, *HuC* and Hel-N1 are each found to have three RNA-recognition motifs and share a homology of as high as 86-90 % with one another. Indispensable for the neuron-specific local expression and the development and maintenance of the nervous system, such proteins are believed to play an important role in neurogenesis and its control in vertebrates, like *Drosophilia elav* protein, when account is taken of the high homology between elav, and HuD, *HuC* and Hel-N1.

In neurogenesis, clusters of cells must be separated and undergo mitosis to develop into differentiated cells, that is, neurons. In this development, the Hu protein may be a useful marker. In the case of zebrafish embryos, *HuC* is expressed at high levels during the whole neurogenesis process, beginning with the first expression in the proneuronal domains of the neural plate (Kim et al., 1996a). Hu proteins which show neuron-specific expression are complementary to other RNA-binding proteins which are encoded by murine *musashi* (Sakakibara et al., 1996). The murine musashi gene is expressed in neural stem cells. When the cells are differentiated to neurons, *musashi* ceases to be expressed, but the expression of Hu proteins starts. While the musashi gene is

responsible for the control necessary for differentiation and the maintenance of mitotic cells, *Hu* genes function to control differentiation-relevant genes and maintain the differentiated cells. In consequence, *musashi* suppresses differentiation whereas *Hu* suppresses proliferation (Okano, 1995). It is inferred that Hu proteins associate with certain domains of RNA through their RNA-binding motifs to control their expression during neurogenesis.

Zebrafish is an important model that provides clues to understanding the early control of neurogenesis in vertebrates because it has a relatively simple nervous system and many genes responsible for a range of early developmental events have been identified. They are particularly well suited to genetic analysis by virtue of the fact that large numbers of embryos can be easily obtained and raised to maturity within a relatively short period. Furthermore the embryos are completely transparent during the first day of development at the time of which their nervous system is established, so it is easy to observe the developmental events.

Identification of the *HuC* promoter is prerequisite to investigate the mechanism in which the neuron-specific expression of *HuC* is controlled. In the present invention, the *HuC* promoter, which is extensively used as a useful tool in the study of

early neurogenesis in zebrafish, was isolated and analyzed so as to study cellular, molecular, and genetic interactions that control early neurogenesis in vertebrates.

The *HuC* promoter provided by the present invention has a transcription start site which starts with G (see Fig. 2). The transcription start site mapped at G is consistent with the report that RNA polymerase II prefers to start at purines (Baker and Ziff, 1981). The presence of one CCAAT box (-64/-60), one GATA-1 (-241/-238) and one SP1 (-213/-208) site were revealed to be present in the immediate upstream region of the transcription start site, suggesting the possibility that the core promoter for *HuC* is located around this region. However, there is no obvious TATA box near the region 30-bp upstream of the transcription start site. The most striking feature of the 5'-flanking sequence of the *HuC* gene is the presence of 18 E-box sequences, which indicates that E-box-binding bHLH transcription factors (Murre et al., 1994) take an important part in the neuron-specific regulation of *HuC* gene expression. This is consistent with the previously suggested role of bHLH transcription factor like *ngn1* in determination of neuronal fate. Additionally, one putative MyT1 binding site, which has also been reported to be essential for neuronal differentiation, was identified at nucleotide

position -2687/-2680.

In another aspect of the present invention, there are provided a fused gene construct in which the *HuC* promoter and genes under the regulation of the *HuC* promoter are combined, and a transgenic animal which harbors the fused gene construct at its genome.

To examine early neurogenesis, extensive attempts have been made using zebrafish mutants in which the distribution of *HuC* mRNA is altered. In this connection, RNA *in situ* hybridization is used to screen the embryos for changes in the distribution of *HuC*-expressing cells. However, this in situ hybridization is disadvantageous in that it is impossible to examine a large number of live embryo mutants not only because embryos are killed by chemicals during the observation of development events, but because the experiment procedure is complicated.

According to the present invention, an embryological method by which changes in the early pattern of neurons can be visibly detected rapidly from live embryos is provided, thereby overcoming the limitation of the conventional RNA in situ hybridization. To this end, the isolated *HuC* promoter was used to create a zebrafish transformant which expresses GFP (green fluorescence protein) in a neuron-specific pattern.

In detail, a fused gene construct in which a *GFP*

gene was located downstream of the *HuC* promoter (*HuCP-GFP*) was microinjected into one-cell stage zebrafish embryos. After two days of growth, embryos which showed neuron-specific expression of GFP were selected under a fluorescence microscope and raised to maturity. The recombinant plasmid in which a *GFP* gene was inserted downstream of the *HuC* promoter, named p*HuC*10GFP, was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the deposition No. KCTC 0802BP on June. 9, 2000. Further, the selected sperm which expresses *GFP* specifically in neurons was deposited with KRIBB under the deposition No. KCTC 0844BP on July 27, 2000.

50 male and female zebrafish adults that had been grown from the embryos for three months, were crossed with wild-type male and female adults, and the progenies were tested for germline transmission of *HuCP-GFP* under the fluorescence microscope. One male adult which had shown GFP expression at an embryo stage was selected as a first-generation transgenic *HuCP-GFP* founder. When the selected transgenic founder male fish was crossed with a wild-type female zebrafish, the frequency at which the *HuCP-GFP* gene was inherited to the F_1 progeny from the first-generation transgenic founder by germline transmission was measured to be 12 %. Upon reaching sexual maturity,

male and female F_1 heterozygous transgenic zebrafishes (*HuCP-GFP*$^{+/-}$) were crossed with each other and approximately 25 % of the F_2 embryos were identified as homozygous *HuCP-GFP* transgenics (*HuCP-GFP*$^{+/+}$) based on the level of GFP expression.

The expression level of GFP in the homozygous transgenic zebrafish was approximately two-fold higher than that in the heterozygous line, and neuron-specific GFP expression in the brain and spinal cord could be easily visualized (Fig. 7). The distribution of neurons in live zebrafish embryos can be visualized using confocal laser microscopy.

GFP transcription in the transgenic zebrafish embryos was detected by in situ hybridization using an antisense GFP RNA probe, at 11 hpf (hours post fertilization), which was close to the time point at which endogenous *HuC* transcripts were first seen in the wild-type zebrafish embryos. In all cases, GFP gene expression was found in the same region near the neural plate. This observation indicates that the neuron-specific expression of GFP in the transgenic zebrafish embryos follows the same pattern in terms of space and time as in the *HuC* transcripts of wild-type zebrafish embryos. Therefore, it was demonstrated that the *HuC* promoter isolated in the present invention is not only identified to comprise the complete regulatory region for the *HuC* gene which directs

neuron-specific expression, but the expression of a GFP gene in the transgenic zebrafish is neuron-specific and shows the same pattern as the *HuC* gene of wild-type zebrafish.

In a further aspect of the present invention, there is provided a method for making the transgenic animal. The method can be broken down into the following five steps:

1) Preparing a fused gene construct in which a *HuC* promoter responsible for neuron-specific expression in zebrafish is ligated to a fluorescence protein gene.

2) Microinjecting the fused gene construct into embryos.

3) Selecting embryos showing neuron-specific expression of GFP.

4) Crossing adults of the selected embryos with wild-type adults to produce F_1 heterozygous transgenic progeny.

5) Self-crossing the F_1 heterozygous transgenic progeny with each other to produce F_2 homozygous transgenics.

In the step 1), the fluorescence protein gene may be selected from the group consisting of genes coding for GFP, luciferase and β-galactosidase. In a preferred embodiment, a recombinant plasmid for stable expression of *GFP* in neurons is constructed which

contains the 5′-flanking region, exon-1, a part of exon-2 and the intervening intron-1 of *HuC*, and a GFP-encoding base sequence. This *HuCP-GFP* fused gene construct, named p*HuC*10GFP, was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the deposition No. KCTC 0802BP on June. 9, 2000.

In still a further aspect of the present invention, there is provided a method for visibly screening mutants whose nervous system is altered, with ease.

Large-scale mutagenesis screening processes have already identified a number of mutants in which the early pattern of neurons is altered. By taking advantage of the transgenic zebrafish of the present invention, living mutants in which the early pattern of neurons is altered can be visibly selected within a short period of time. The success in screening such mutants reflects not only the value of *HuC* as an early neuronal marker, but also that its promoter and the transgenic zebrafish created by using it are useful as a tool in the study on neurogenesis in vertebrates.

The method for screening neurogenesis mutants according to the present invention comprises the steps of:

1) crossing a homozygous zebrafish which harbors a *HuCP-GFP* fused gene construct in its genome with an

unknown heterozygous neurogenesis mutant to produce F_1 progeny;

2) back-crossing F_1 progeny with the unknown heterozygous neurogenesis mutant to obtain homozygous neurogenesis mutants; and

3) comparing the GFP fluorescence between the homozygous neurogenesis mutant embryos and the F_1 progeny embryos.

To illustrate the usefulness of the screening method, the *HuCP-GFP* gene was introduced into mib (mind bomb) mutant zebrafish (Schier et al., 1996) which is characterized by a neurogenic phenotype with supernumerary early differentiating neurons and a deficiency in late differentiating neurons. In one preferred embodiment, homozygous *HuCP-GFP* transgenic zebrafish (*HuCP-GFP*$^{+/+}$) were crossed with heterozygous mib carriers (mib$^{+/-}$). Upon reaching sexual maturity, the resulting F_1 progeny (*HuCP-GFP*$^{+/-}$/mib$^{+/-}$) were back-crossed with the heterozygous mib mutant (mib$^{+/-}$) to obtain *HuCP-GFP*$^{+/-}$/mib$^{-/-}$ mutant embryos. Making neuronal hyperplasia evident in *HuCP-GFP*$^{+/-}$/*mib*$^{-/-}$ transgenic embryos, much more intense GFP fluorescence was observed in those transgenic embryos under a fluorescence microscope, compared to *HuCP-GFP*$^{+/-}$ embryos. These results reflect how the screening method using the *HuCP-GFP* transgenic zebrafish could be used for isolating and analyzing neurogenesis mutants in living

zebrafish with ease.

EXAMPLES

A better understanding of the present invention may be obtained in light of the following examples which are set forth to illustrate, but are not to be construed to limit the present invention.

EXAMPLE 1: Early Neuronal Expression of *HuC* in Zebrafish Embryo

In a previous study, *HuC* was revealed to be a useful marker for neurons in zebrafish based on the fact that it is expressed in nascent primary neurons soon after gastrulation (Kim et al., 1996; Park et al., 2000). In this example, to provide additional evidence that *HuC*-positive cells are early neurons, the expression of *HuC* was compared with that of *Delta*B, which has recently also been disclosed to be expressed in nascent neurons by recent studies (Haddon et al). With reference to Fig. 1, there are fluorescence photographs taken of dorsal parts of embryos, showing the comparison of *HuC* and *Delta*B mRNA expression in the neural plate at the 3-somite stage. As shown at the sites of ps (primary sensory neuron), pin (primary intermediate neuron) and pmn (primary motor neuron) of

the photographs, the expression of *HuC* (A) in three longitudinal columns within the neural plate is very similar to that of *Delta*B (B) at the 3-somite stage.

EXAMPLE 2: Isolation and Characterization of 5'-Flanking Region Containing Promoter for *HuC* Genomic DNA

In order to isolate the zebrafish *HuC* promoter region, a zebrafish genomic library was screened through hybridization using a radiolabeled probe derived from the 5'-UTR of zebrafish *HuC* cDNA (Kim et al., 1996). First, a zebrafish genomic DNA library (Clontech) was screened with [α-^{32}P]dCTP-labeled cDNA fragments containing the 5'-UTR of zebrafish *HuC* cDNA. A number of positive clones were identified by plaque hybridization. Of them, two clones containing a 15-kb *Not*I (clone 4) and a 18-kb *Not*I (clone 8) genomic DNA insert, respectively, were purified to single phage plaques. Preliminary restriction analysis and partial nucleotide sequencing resulted in the finding that a 7-kb *Nco*I DNA fragment of the 15-kb *Not*I genomic insert contained a 5-kb sequence upstream of the translation start codon ATG after the subcloning of the *Nco*I fragment into plasmid pGEM7(+) (Promega). To narrow the putative promoter region to a more defined one, an internal *EcoR*I fragment containing a 3.2-kb

upstream sequence from the translation start codon ATG was isolated, followed by analyzing its complete nucleotide sequence by the dideoxynucleotide chain termination method (Sanger et al., 1977).

The transcription start site in the 5′-UTR of *HuC* cDNA, which was analyzed to have Sequence No. 1, was determined by primer extension using an antisense oligonucleotide derived from the 5′-UTR sequence.

Using T4 polynucleotide kinase (Promega), an oligonucleotide primer of Sequence No. 2 derived form the exon-1 of the zebrafish *HuC* gene was end-labeled with [γ-^{32}P]ATP (Amersham) to 10^8 cpm/μg. 60 μg of total RNA isolated from each of 24-hpf zebrafish embryos and yeast tRNA were hybridized with the isotope-labeled primer (5×10^5 cpm) at 30 °C. After 18 hours of incubation, the reactions were precipitated by ethanol and resuspended in 20 μl of a reverse-transcriptase reaction mixture (50 mM Tris-Cl, 6 mM $MgCl_2$, 40 mM KCl, 10 mM dithiothreitol, pH 8.5). An AMV reverse transcriptase (Boehringer Mannheim) was added at an amount of 200 units to the reactions which were then incubated at 42 °C for 1 hour. After being precipitated in ethanol, the cDNA products were electrophoresed on 6 % polyacrylamide gel containing 8 M urea. To map the nucleotide position for the transcription start site, a separate DNA sequencing reaction using a 3.6-kb *EcoRI* fragment of zebrafish

HuC genomic DNA with the same oligonucleotide primer was performed and subjected to electrophoresis.

With reference to Fig. 3, there is shown an autoradiograph in which the transcription initiation site of the *HuC* gene is determined by primer extension. The Z lane is for the 24 hpf zebrafish embryos (Z) while the Y lane is for the yeast tRNA. An extended cDNA band from zebrafish RNA is indicated by the arrow and the corresponding nucleotide G is marked by an asterisk. As shown in this autoradiograph, a single cDNA band was extended on a template mRNA derived from 24-hpf zebrafish embryos. Using this cDNA, the nucleotide position of transcription initiation site was mapped within the genomic DNA and referred to as +1, and all subsequent nucleotide positions were numbered relative to this location, as shown in Fig. 2. The transcription initiation site mapped at G is consistent with the report that RNA polymerase II prefers to start at purines (Baker and Ziff, 1981).

To analyze the zebrafish *HuC* promoter, an examination was made of the GFP expression patterns in the neuron, muscle and other tissues of embryos by use of various deletion constructs. With reference to Fig. 4, there are shown structures of the zebrafish deletion constructs, along with their transient expression patterns. As seen in the schematic diagram of Fig. 4, a 3.6-kb *Eco*RI fragment of zebrafish *HuC*

genomic DNA was identified to consist of 2,771 bp of the 5′-upstream sequence, 391 bp of exon-1 (382-bp 5′-UTR followed by a 9-bp coding sequence), and 429 bp of a part of intron-1 on the basis of the transcription initiation site and a previously reported *HuC* cDNA sequence. Analysis of the nucleotide sequence for the region immediately upstream of the transcription start site revealed the presence of one CCAAT box (-64/-60), one GATA-1 (-242/-238), and one SP1 (-213/-208) site, suggesting the possibility that the core promoter *HuC* is located around this region. However, there was no obvious TATA box near the region 30-bp upstream of the transcription initiation site. The most striking feature of the 5′-flanking sequence of the *HuC* gene is the presence of as many as 18 E-boxes as shown in Fig. 2, which indicates an important role for E-box-binding bHLH transcription factors in the neuron-specific regulation of *HuC* gene expression (Murre et al., 1994). This agreed with the previously suggested role of bHLH transcription factors such as *ngn1* in the determination of neuronal fate. Furthermore, one putative MyT1 binding site, which has also been reported to be essential for neuronal differentiation, was identified at the nucleotide position -2687/-2680 as shown in Fig. 2.

EXAMPLE 3: Identification of 5′-Flanking Region for

Neuron-Specific Expression of *HuC* Gene

An examination was made to determine the size of the 5'-upstream sequence, containing the putative *HuC* promoter region, in the 3.6-kb *EcoRI* fragment, which is sufficient to restrict the expression of the GFP reporter gene to neurons.

First, a 3.2-kb (-2771/+382) genomic DNA fragment amplified by PCR from a template of the 3.6-kb *Eco*RI genomic DNA fragment, was fused with the GFP-encoding sequence of the plasmid pEGFP-1 (Clontech) at the *Eco*RI/*Sma*I site to construct a *HuCP-GFP* gene, designated ΔEco. The PCR was performed using pfu Turbo DNA polymerase (Stratagene).

The ΔEco DNA construct was microinjected into zebrafish embryos at the one-cell stage and its control in gene expression was analyzed by observing the GFP expression in the embryos under a fluorescence microscope. At 48 hpf, embryos microinjected with ΔEco were found to express GFP in all regions of the nervous system. The results are given in Fig. 5. As shown in the fluorescence photographs of Fig. 5, the telencephalic cluster, the retinal ganglion neuron, the trigeminal ganglion neuron, medial longitudinal fasciculus and dorsal longitudinal fasciculus are the sites in which GFP was most easily observed. Also, the peripheral projections of Rohon-Beard neurons as well

as their central projections that terminate in the hindbrain could be easily identified by the strong fluorescence of GFP. Additionally, the major axonal tracts that make up the early axonal scaffold in the brain were visualized by the strong GFP expression in axons.

Furthermore, the neuronal specificity of the GFP expression driven by the ΔEco was identified again in whole mounts with an anti-GFP polyclonal antibody, indicating that the 5′-flanking promoter region in the ΔEco construct contains all regulatory elements necessary to restrict *HuC* gene expression to the neurons.

EXAMPLE 4: Functional Analysis of *HuC* Promoter in Zebrafish Embryos

For the identification of regulatory regions necessary to maintain *HuC* gene expression exclusively in the neurons, serial deletions of the 5′-flanking region in the ΔEco construct were generated from both 5′- and 3′-ends, as shown in Fig. 4.

To this end, first, the ΔEco construct was cleaved with *Eco*RI/*Hind*III, *Eco*RI/*Sph*I, *Eco*RI/*Kpn*I, *Eco*RI/*Bst*XI and *Eco*RI/*Sac*I. Thereafter, larger DNA fragments from each of the restriction reactions were isolated and self-ligated to yield ΔHind (-2473 to

+382 bp), ΔSph (-1962 to +382 bp), ΔKpn (-1161 to +382), ΔBst (-431 to +382) and ΔSac (-251 to +382) constructs. Separately, the ΔEco construct was also digested with *Eco*RI/*Kpn*I, *Eco*RI/*Bst*XI, and *Eco*RI/*Sac*I, and the smaller DNA fragments were inserted into the compatible sites in plasmid pEGFP-1. When appropriate restriction sites were not available, 3'-ends were blunted with klenow enzyme and inserted into the *Eco*RI/*Sma*I site. The CCAAT-box sequence in the ΔSac construct was mutated to CCCAT by site-directed mutagenesis using a site-directed mutagenesis kit (Stratagene) with the oligonucleotide primer of Sequence No. 3 to give a ΔSac-M construct.

Changes in GFP expression resulted from the deletions were identified by examining GFP expression at 48 hpf in embryos injected with specific deletion constructs at the one-cell stage. The results are shown in Fig. 6.

When embryos were injected with the ΔHind construct (-2473/+382), the expression of GFP in neurons was similar to that with the ΔEco construct. The GFP expression, however, was also observed in muscle cells (Fig. 6A). This result suggests the role of a putative MyT1 binding site (-2687/-2680) and/or two E-box sequences (17^{th} at -2565/-2560 and 18^{th} at -2665/-2650) (Figs. 2 and 4) in the suppression of *HuC* expression in muscle cells. Since MyT1 is not

expressed in muscle cells, it is more likely that loss of the E boxes in this deletion mutant leads to the more promiscuous expression of GFP.

When the 5′-flanking region of the *HuC* promoter was progressively deleted toward the 3′-end, GFP expression was increased only in muscle cells without concomitant loss of GFP expression in neurons. That is, the expression intensity of GFP in muscle cells increased in the order of the microinjection with constructs ΔHind (-2473/+382), ΔSph (-1962/+382), ΔKpn (-1162/+382), and ΔBst (-431/+382). Finally, GFP expression in muscle cells driven by the ΔBst construct increased to the extent of overwhelming its expression in neuronal cells as shown in Fig. 6B. These results indicate that the 12 E-box sequences (5-16) play a more important role in the suppression of *HuC* expression in muscle cells than in the neuron-specific expression of *HuC*.

In contrast, the ΔSac (-251/+382) construct drives ubiquitous expression of GFP in all tissues, including skin and notochord and neurons, of most embryos, giving the suggestion that the proximal three E-boxes present in the ΔBst construct are indispensable for the maintenance of neuron-specific expression of *HuC* as shown in Figs. 6C and 6D.

To test the function of the putative CCAAT-box, a point mutation was introduced into the ΔSac construct

to change the first A to C. The resulting ΔSac-M construct was found to almost completely lose its promoter activity, as illustrated in Fig. 4. Therefore, a 5′-flanking region spanning 251 bp in the ΔSac construct was proved to represent a core promoter for the *HuC* gene.

This result, that is, the localization of a core promoter region within the ΔSac construct, was confirmed by testing GFP expression with ΔEbst (-2771/-431), ΔEkpn (-2771/-1162), and ΔEsac (-2771/-251) constructs, which all lack the 251-bp 5′-flanking region of the ΔSac construct. Embryos injected with ΔEbst (-2771/-431), ΔEkpn (-2771/-1162) and ΔEsac (-2771/-251) constructs did not show any significant GFP expression, supporting the role of the 251-bp 5′-flanking sequence as the core promoter for the zebrafish *HuC* gene. In addition, these results indicate that 17 E-box sequences and one MyT1 binding site, along with the proximal core promoter region, orchestrate the neuron-specific expression of *HuC*.

EXAMPLE 5: Creation of Transgenic Zebrafish Capable of Neuron-Specific Expression of GFP

5-1: Construction of fused gene

For the stable expression of GFP in neurons, a fused gene construct (hereinafter referred to as "*HuC*

promoter-GFP' or "*HuCP-GFP*") was prepared consisting of exon-1, intron-1, a part of exon-2, and a GFP-encoding sequence.

Using the clone #4 which harbors the 15-kb genomic DNA fragment prepared in Example 2, the *HuCP-GFP* fused gene was constructed as in the following consecutive recombination processes. To begin with, plasmid pEGFP-C1 DNA was double-digested with *Eco*47III/*Xho*I, followed by inserting the resulting 0.75-kb GFP DNA digest into the StuI/XhoI site of the plasmid vector CS2A(-) which was previously derived from the self-ligation of the large fragment remaining after the removal of the CMV promoter when plasmid CS2(-) was digested with *Sal*I/*Hind*III. The resulting recombinant plasmid CS2A(-)-GFP was further cleaved with NcoI, after which the *HuC* promoter containing, 10.5-kb NcoI digest from the 15-kb *HuC* genomic DNA of clone #4, which contains 4.6 kb of the 5'-flanking region, 391 bp of exon-1, 5.5 kb of intron-1, and 15 bp of exon, was inserted into the NcoI site of the recombinant plasmid CS2A(-)-GFP so that the GFP gene was regulated under the *HuC* promoter. In addition, this insertion brought about the effect of newly replacing the translation initiation codon ATG of the GFP gene which was lost upon the excision of the GFP gene from the plasmid. Finally, the resulting recombinant plasmid CS2A(-) containing the 10.5-kb *HuC*

gene and the 0.75-kb GFP gene was further digested by *Eco*RV/*Bam*HI to remove the 0.5-kb *Eco*RV/*Nco*I DNA fragment at the most 5'-upstream sequence of the 5'-flanking sequence in the 4.6-kb *HuC* genomic DNA, and then self-ligated to construct a *HuCP-GFP* fused gene expression plasmid. This resulting recombinant expression vector was linearized by a single-cut restriction enzyme *Sca*I and the linearized forms of DNA were microinjected into one-cell stage embryos. The recombinant plasmid pHuC10GFP, which contains the *HuCP-GFP* fused gene construct, was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the deposition No. KCTC 0802BP on June. 9, 2000.

5-2: Preparation of zebrafish embryos

Zebrafish were raised at 28 °C with cycles of 14 hours in the daylight and 10 hours in the dark. Until the time of crossing, male and female were grown in separate tanks. Upon mating, beads were laid sufficiently to completely cover the bottom of the incubation bath lest the adults eat the eggs. Under a light, the fertilized eggs were harvested at appropriated intervals of 1-2 hours with the aid of a tube. After being raised for 2-4 days in incubation water containing 60 μg/ml of sea salts (Sigma), the

embryos microinjected with the recombinant plasmids and control embryos were transferred to a common water bath for growth. Zebrafish were maintained with care according to a well-known process (Westerfield, 1995).

5-3: Preparation of embryos microinjected with *HuCP-GFP* fused gene construct

The recombinant plasmid CS2A(-) DNA containing the *HuCP-GFP* fused gene construct was microinjected into 500 one-cell stage zebrafish embryos. 48 hours after microinjection, embryos which transiently expressed GFP in neurons were identified by fluorescence microscopy and raised to sexual maturity.

For use in microinjection, the fused gene construct was prepared using EndoFree Plasmid kit (Qiagen). In this regard, the *HuCP-GFP* fused gene expression plasmid was linearized with an appropriate restriction enzyme and isolated through the extraction in phenol-chloroform and the precipitation by ethanol. Zebrafish embryos were stored in plastic vessels with a diameter of 10 cm and microinjected with DNA in advance of the first cleavage under a dissecting microscope. For microinjection, DNA concentration was adjusted to 100 μg/ml in 0.1 M KCl solution (Stuart et al., 1990) containing 0.5 % phenol red, and the solution with such a DNA concentration was injected

into the one-cell stage embryos at an amount of 100-200 pl per embryo prior to the first cleavage.

Adults were crossed with wild-type fish and progeny were tested for germline transmission of *HuCP-GFP* under a fluorescence microscope. One male adult capable of germline transmission was identified as a transgenic *HuCP-GFP* founder fish and back-crossed with wild-type female fish. As a consequence, twelve percent of the F_1 progeny was found to inherit the *HuCP-GFP* gene by germline transmission from the founder. Upon reaching sexual maturity, male and female F_1 heterozygous transgenic zebrafish were crossed with each other to yield F_2 progeny, approximately a quarter of which were identified as homozygous transgenic zebrafish (*HuCP-GFP*$^{+/+}$). The selected sperm of the homozygous transgenic zebrafish microinjected with the plasmid pHuC10GFP capable of directing the neuron-specific expression of GFP were deposited with KRIBB under the deposition No. KCTC 0844BP on July 27, 2000.

EXAMPLE 6: Identification of Regulation Pattern of *HuC* Promoter in Transgenic Zebrafish Neuron

An examination was made of the *HuC* promoter-driven GFP expression in neurons of the *HuCP-GFP* transgenic zebrafish prepared in Example 4. The

expression level of GFP in the homozygous transgenic zebrafish was approximately two-fold higher than that in the heterozygous line, and neuron-specific GFP expression in the brain and spinal cord could be easily visualized, as shown in Fig. 7. Additionally, the *HuCP-GFP* transgenics made it possible to visualize the detailed distribution of neurons in live zebrafish embryos under a confocal laser microscope. In detail, at approximately 24 hpf, clear GFP expression could be identified in primary commissural neurons, Rohon-Beard neurons and motorneurons of the spinal cord by their bright fluorescence, showing in detail the precise positions of neurons, according to type (Fig. 7D).

In order to determine whether the spatial and temporal GFP expression in the *HuCP-GFP* transgenic zebrafish is similar to the spatial and temporal expression of *HuC* mRNA in wild-type zebrafish embryos, RNA in situ hybridization was conducted as follows. First, an antisense digoxigenin-labeled RNA probe for the 3'-UTR of zebrafish *HuC* cDNA was produced using a DIG-RNA labeling kit (Boehringer Manheim), followed by performing hybridization and detection with an antidigoxigenin antibody coupled to alkaline phosphatase according to the instruction of Jowett and Lettice (Jowett and Lettice, 1994).

By RNA in situ hybridization using the antisense GFP RNA probe, the GFP transcription in the transgenic

zebrafish embryos was detected at 11 hpf, which was close to the time point at which endogenous *HuC* transcripts were first seen in the wild-type zebrafish embryos (Figs. 8A and 8B).

For the examination of the neuronal specificity of GFP expression in the *HuCP-GFP* transgenic lines, GFP-positive cells in the transgenic zebrafish embryos were visualized by a whole-mount immunostaining method using an anti-GFP polyclonal antibody.

In more detail, dechorionated embryos were fixed in BT buffer (0.1 M $CaCl_2$, 4 % sucrose in 0.1 M $NaPO_4$, pH 7.4) containing 4 % paraformaldehyde for 12 hours at 4 °C, and then rinsed in PBST (1xPBS, 0.1 % Triton X-100, pH 7.4). After being frozen in acetone at -20 °C for 7 min, the embryos were washed three times with PBST, and immersed for 1 hour in a PBS-DT blocking solution. (1x pBST, 1% BSA, 1% DMSO, 0.1% Triton X-100, 2% goat serum). Then, the embryos were reacted with 1:1000 diluted anti-GFP polyclonal antibody (Clonetech) for 4 hours at room temperature, washed 10 times for 2 hours with PBS-DT, and incubated with 1:500 diluted biotinylated goat anti-rabbit antibody (Vector) at 4 °C overnight. The embryos were washed for 6 hours in PBS-DT, incubated for 2 hours at room temperature in Vectastain Elite ABC reagent (Vector), washed five times in PBS-DT, and washed three times in 0.1 M $NaPO_4$. Afterwards, the embryos were incubated

with 1 ml of DAB solution (1% DMSO, 0.5 mg/ml diaminobenzidine, 0.0003% H_2O_2 in 0.05 M $NaPO_4$, pH 7.4) at room temperature. When a color change was observed while monitoring the embryos for 5 to 10 min under a dissecting microscope, the chromogenic reaction was stopped by the addition of a 0.1 M $NaPO_4$ solution (pH 7.4).

Patterns of whole-mount in situ hybridization patterns and immunostaining were observed using a Zeiss Axiocop microscope. Embryos and adult fish were anesthetized using tricaine (Sigma) according to the instruction of Westerfield (1995), and examined through an FITC filter on a Zeiss Axioskop fluorescence microscope. Laser confocal microscopic images were obtained using Leica DM/R-TCS laser scanning microscope equipped with an FITC filter.

In 24-hpf transgenic zebrafish embryos, various neurons, including GFP expression in telencephalic cluster, anterior commissure, epiphyseal cluster, posterior commissure, tract of posterior commissure, postoptic commissure, tract of the postoptic commissure, olfactory placodes, nuclei of medial longitudinal fasciculus, medial longitudinal fasciculus, trigeminal ganglion, seven rhombomeres in the hindbrain, were recognized by the anti-GFP antibody as shown in Fig. 8. Further, early motorneurons, Rohon-beard neurons and interneurons of

the spinal cord were also detected by the anti-GFP antibody in the same GFP expression pattern as that observed under the laser confocal microscope. These results indicate that the GFP RNA expression in the transgenic line is temporally and spatially similar to that of *HuC* mRNA transcripts in the wild-type zebrafish.

EXAMPLE 7: Characterization of Neurogenesis Mutant Using *HuCP-GFP* Transgenic Zebrafish

With the aim of identifying the usefulness of *HuCP-GFP* transgenic zebrafish as a useful tool for characterizing neurogenesis mutants, the *HUCP-GFP* gene was introduced into the *mib* mutant zebrafish (Schier et al., 1996). The mib mutant is known as a neurogenic phenotype of neural hyperplasia, in which supernumerary early differentiating neurons exist.

To begin with, homozygous *HuCP-GFP* zebrafish ($HuCP\text{-}GFP^{+/+}$) were crossed with heterozygous mib carriers (mib+/-). Upon reaching sexual maturity, the resulting F_1 progeny ($HuCP\text{-}GFP^{+/-}$/mib$^{+/-}$) were back-crossed with the heterozygous min carriers ($mib^{+/-}$) to yield heterozygous mutant embryos ($HuCP\text{-}GFP^{+/-}/mib^{+/-}$). Not only much more intense GFP fluorescence, but also more extensive GFP expression regions were detected in the F_2 heterozygous mutant embryos ($HuCP\text{-}GFP^{+/-}/mib^{+/-}$)

than in the heterozygous transgenic embryos (*HuCP-GFP*+/-), demonstrating that neuronal hyperplasia occurs in *HuCP-GFP*$^{+/-}$/*mib*$^{+/-}$ transgenic embryos, as shown in Fig. 9. Therefore, the *HuCP-GFP* transgenic zebrafish of the present invention can be useful for isolating and analyzing neurogenesis mutants in zebrafish.

INDUSTRIAL APPLICABILITY

As described hereinbefore, the *HuC* promoter, whose expression is a useful early marker for neurons in zebrafish, is isolated and characterized for base sequence, regulatory element, and neuron-differentiating mechanism, in accordance with the present invention. Also, the present invention provides a transgenic zebrafish line that expresses GFP specifically in neurons. In addition, the *HuC* promoter of the present invention can be used in the study of the regulatory mechanism responsible for the differentiation of the nervous system. Taken together, these results indicate that the *HuCP-GFP* transgenic zebrafish of the present invention enable the direct identification of neurogenesis and axonogenesis, as well as being a valuable tool for isolating and analyzing neurogenesis mutants in live zebrafish with ease.

The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation. Many modifications and variations of the present invention are possible in light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : HUH, Tae-Lin
Department of Genetic Engineering, College of Natural Sciences,
Kyungpook National University, Taegu 702-701,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: **pHuC10GFP**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 0802BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **June 09 2000.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s): BAE, Kyung Sook, Director Date: **June 15 2000**

Form BP/4 (KCTC Form 17) sole page

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

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TO : HUH Tae-Lin
Department of Genetic Engineering, College of Natural Sciences,
Kyungpook National University, Taegu 702-701,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
zf HuCP10GFP sperm	**KCTC 0844BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **July 27 2000.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or authorized official(s): Bae K.S. BAE, Kyung Sook, Director Date: **August 22 2000**

Form BP/4 (KCTC Form 17)

What is claimed is:

1. A *HuC* promoter with its 5′-flanking region, capable of driving gene expression specifically in neurons.

2. The *HuC* promoter with its 5′-flanking region as set forth in claim 1, wherein the *HuC* promoter has the base sequence listed in Sequence No. 1.

3. A recombinant plasmid p*HuC*10GFP, deposited under the deposition No. KCTC 0820BP, in which a green fluorescence protein (GFP) gene is ligated to the *HuC* promoter of Claim 1.

4. A sperm of a homozygous transgenic zebrafish, deposited under deposition No. KCTC 0844BP, containing the recombinant plasmid of claim 3.

5. A zebrafish, which harbor the recombinant plasmid of claim 3 in their genome and show neuron-specific expression of GFP.

6. A method for generating a transgenic animal, comprising the steps of:

preparing a fused gene construct in which a *HuC* promoter responsible for neuron-specific expression in

zebrafish is ligated to a fluorescence protein gene;

microinjecting the fused gene construct into embryos;

selecting embryos showing neuron-specific expression of GFP;

crossing adults of the selected embryos with wild-type adults to produce F_1 heterozygous transgenic progeny; and

self-crossing the F_1 heterozygous transgenic progeny with each other to produce F_2 homozygous transgenics.

7. The method as set forth in claim 6, wherein said fluorescence protein gene is selected from genes coding for GFP, luciferase and β-galactosidase.

8. The method as set forth in claim 6, wherein said fused gene construct contains the 5′-flanking region, exon-1, a part of exon-2 and the intervening intron-1 of *HuC*, and a GFP-encoding base sequence.

9. The method as set forth in claim 6, wherein said transgenic animal is zebrafish.

10. A method for screening neurogenesis mutants in zebrafish, in which the transgenic zebrafish of claim 5 is utilized.

11. The method as set forth in claim 10, in which the method comprises the steps of:

crossing a homozygous zebrafish which harbors a *HuCP-GFP* fused gene construct in its genome, with an unknown heterozygous neurogenesis mutant to produce F_1 progeny;

back-crossing F_1 progeny with the unknown heterozygous neurogenesis mutant to obtain homozygous neurogenesis mutants; and

comparing the GFP fluorescence between the homozygous neurogenesis mutant embryos and the F_1 progeny embryos.

12. A method for analyzing alterations in the nervous system, in which the transgenic zebrafish of claim 5 is utilized.

FIGURES

FIG. 1

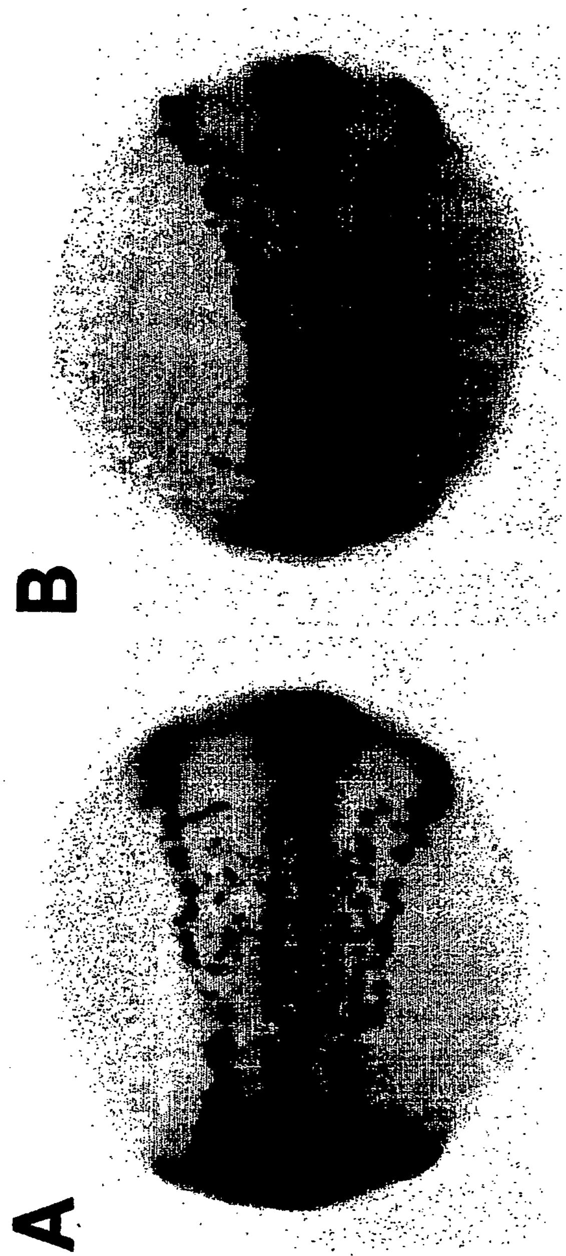

FIG. 2

-2771 GAATTCACTAATTTGAATTTAAATGCATTATCTTTCTATTCCTAAAGACCTTGGGTGACCAAAATCTTAT
-2701 TTTAATAAATAAAACTGTTTATTAAAACTTTTTTGTTTCAAAGAACCATATGTATAGTGAAATTTATAAA
MyT1 E-box
-2631 AATATCAATTTTTAAAAAGCTGGTGTACTCATTTATGTTATGAACTCTAAAACCATATACTGACTGCAAG
-2561 TGATGATGTATAGAGTGATGTTTACGAGTAAACATATTTAGTTGTATACATCCTACTGAGCACATTTTGA
-2491 TGTATGAAATAACATTACAAGCTTTACCCAAATTAAGCCATTTTAAAACACTGCCAATTGAAAATACAAA
-2421 TCCTGGAAAAAATCGTCTTTAGCGCAGTCATTTGAGCCATCCTAATCCGTTACCTCAGACCATAATAAGA
-2351 AGGGATAACACTAGCTGTAGCAATGGAACACATCTGTTTCACACAATCATATCTCCTGCGCCGGTGCTAA
-2281 GCAGATTCAGCGTGATCATAACATGCTTTCCACTCATAAATGTAAATTTACAATTTGCACATGTAAAACA
-2211 GACACTTTTGAGATATTGGATAAAAAAACAAGAGTATATTGCTTAGTTTCATCCACCAGTCATCCCCACA
-2141 GCGTTTGGAAGGCCATAAAAAGTGTCTAAAATCAATGATCATTGAAAGAGCACAAGAGAGACTCTTACGC
-2071 TGTAATGCCACTGGGGACAAAAGTGACAGTCTCTTAATGGGCTCTTCTGGAGGGGCTCCTGAACATTAAA
-2001 AATTATCAGCGAAATTACCGAAAGAGCTTCAAGCAACTGGCATGCTTGATCCTCTGCGTCGGGGCGGTGA
-1931 ATAGGTGCTTCAGATGCCCTCTTACCCACGGGCTGGATTCAGCTGCCCCGCTACCAGCGGAGACCCCCTA
-1861 ATGAGCCTCTGCAATTAAGTTTATTCATGTTAAGTGTGAACGGGGTGCGTGCGGAACTGTGGGCAGCTAA
-1791 CAGACCTGGGTTCTTTGTGCCACAAGTGCTGCCTTTATTCGGCTCACAAAGCAGAAAACAACACCCGCAC
-1721 CTATAATGGCGCCCTCGGCTGGGTCTAAGAAACGTGGCGAGTTGACAGAGCAGAGTGGGCGGGGTTAAGA
-1651 CAGACTGACAGCGGGACCCATCTCCATCCTCTTATTAACGCTTAACGAGTGCCTTCCTCATGCAATATTC
-1581 ATCGCCACTAATATCATCCAAGCTCTGAGCTGAGCTGGCCACTTATGTAAGGCAATTATGTAAAATATCA
-1511 GACAGGGCCCACACTCAGAATCTGACTGGGGTAGAGACGCGGGACGAGAACCGAGAGCAAGAACTGAAAG
-1441 TGAAAGTGACCACTAAAGGGAGGAGAGGACAGAGGGGCAGGATGTGTCAAGATTACCAGAGAACACTTGG
-1371 CCAGAAATGCGCAACCATTGGAGCTCTCCGGATTACCCAAAGGTTAACGAGTTTGAACGCCTCTGCCCAC
-1301 TCGCCATCTCTGATGGTTTCCCAAGAACTCCTCAAGCAAAATATATATAATTGTGTGTATTATGCACAGA
-1231 CACGAGAAAATGCTGTTTTTCTGATCTGCATTACAGCACATTTGCCCGCCAACGACAATACCACCCACTC
-1161 GGTACCTCGCTGACTCCTGATGCCTGATACCTGCGCGGTGACTGTCTACAATCTGCATAATCAAGAGAAG
-1091 TTGTGTTGAAGACGAGCGCCACACAACCGTTTCCACAAGGTCACCCAAGGCCGGTGCAGATGTAGGTGAG
-1021 GTCTCCATAAACAGACTGAAATAAACACATCCTCCGCTGGGAACAACAACCCCCTCACGCCTCATGCATT
-951 CCATAAGCCTACATGCATCTCTTCCAACTTATGGAGACTCGCACCTACCAACATCCGCACAACAAAGATA
-881 TACAGAGCGCGCTCCCTCAGGTCAAGGCCTGTGGGGTCTGTGCAGAAATAGGTCATTTGTCACACATCA
-811 AGTCCTGGGGCAGGAGATGCATTATAGATGAGACCAAACAGCCTGTCTCGGTGAGCTCTACCCACTCCCT
-741 GAGACTAGAAATGGGGGAAGGGAGCTTGAGATAACAACCGCTGCAATCACTGTGTCGATGTTAATATCAG
-671 CACCAACCGGGAACAATAAGGAGATGGATGCATTCATGTTCACATCTTACCAGTCAAGTATCATCGAACC
-601 GGCTTGATAACCACACCTCGTGTAATAGCTGAGCAGATAGTTGTCATTTTAAAGCGTTGGCCTTTGTCGA
-531 TTATGTAATGCGCACATTCAACACATGGTAATATAGAAACGGTTATGTCGAGGTTGTTTTGTCCAGAGAT
-461 GACCTTCACACAGTTACAGCCGCTCTGCATCCAGACAAATGGAGGACTTAATCGTGGACTGCATTCTTAG
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FIG. 3

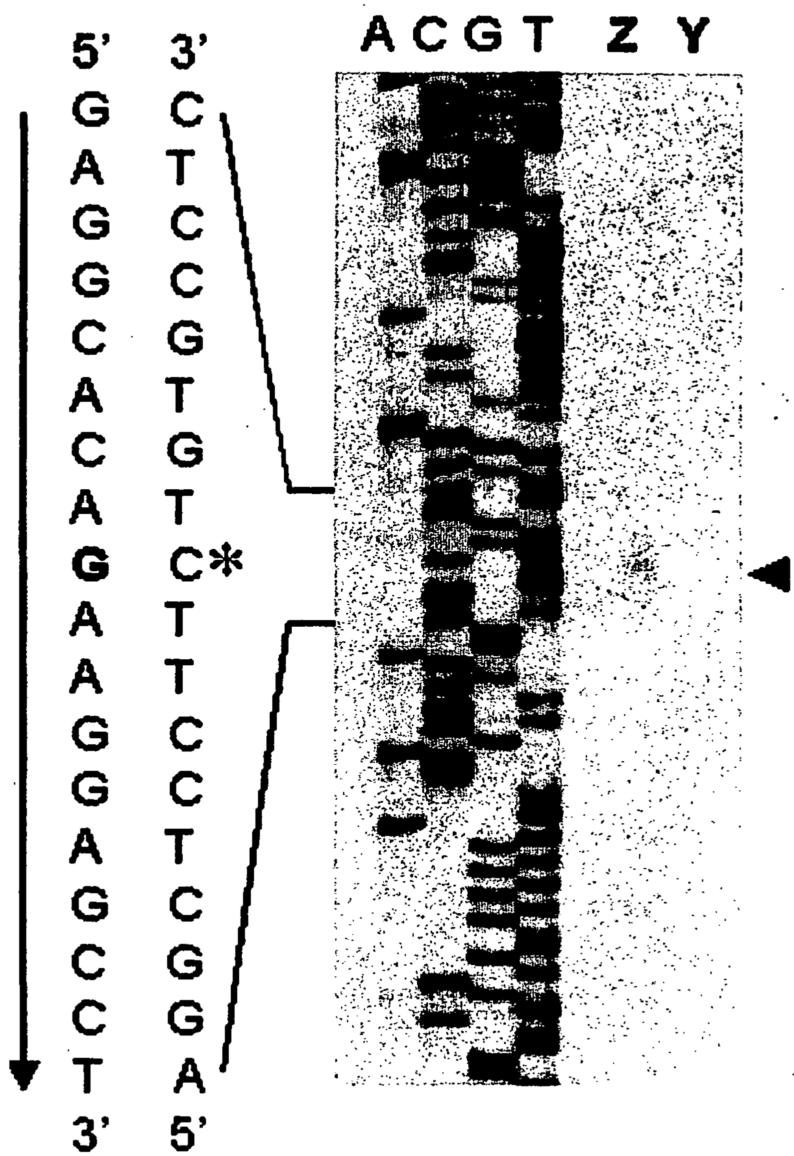

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FIG. 4

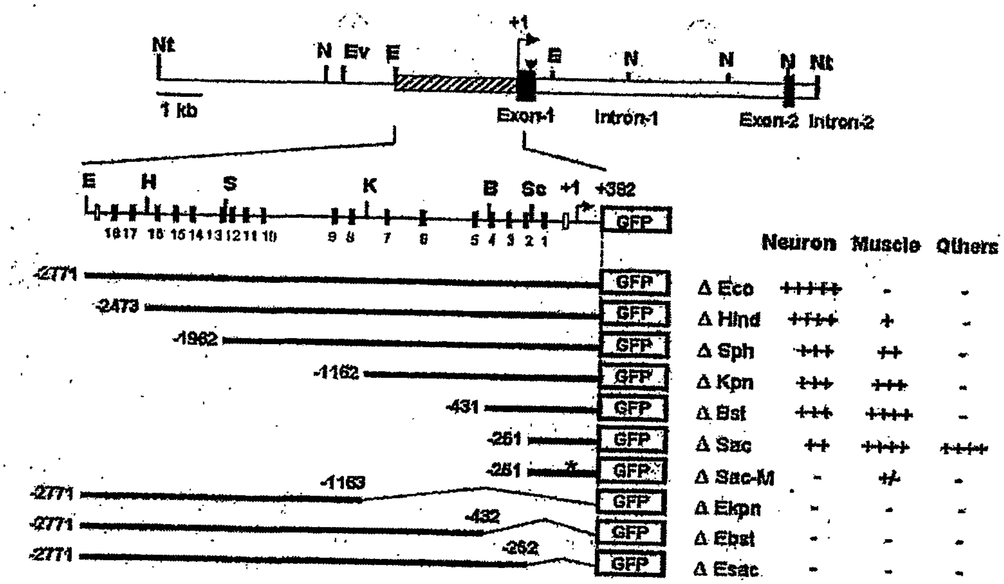

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FIG. 5

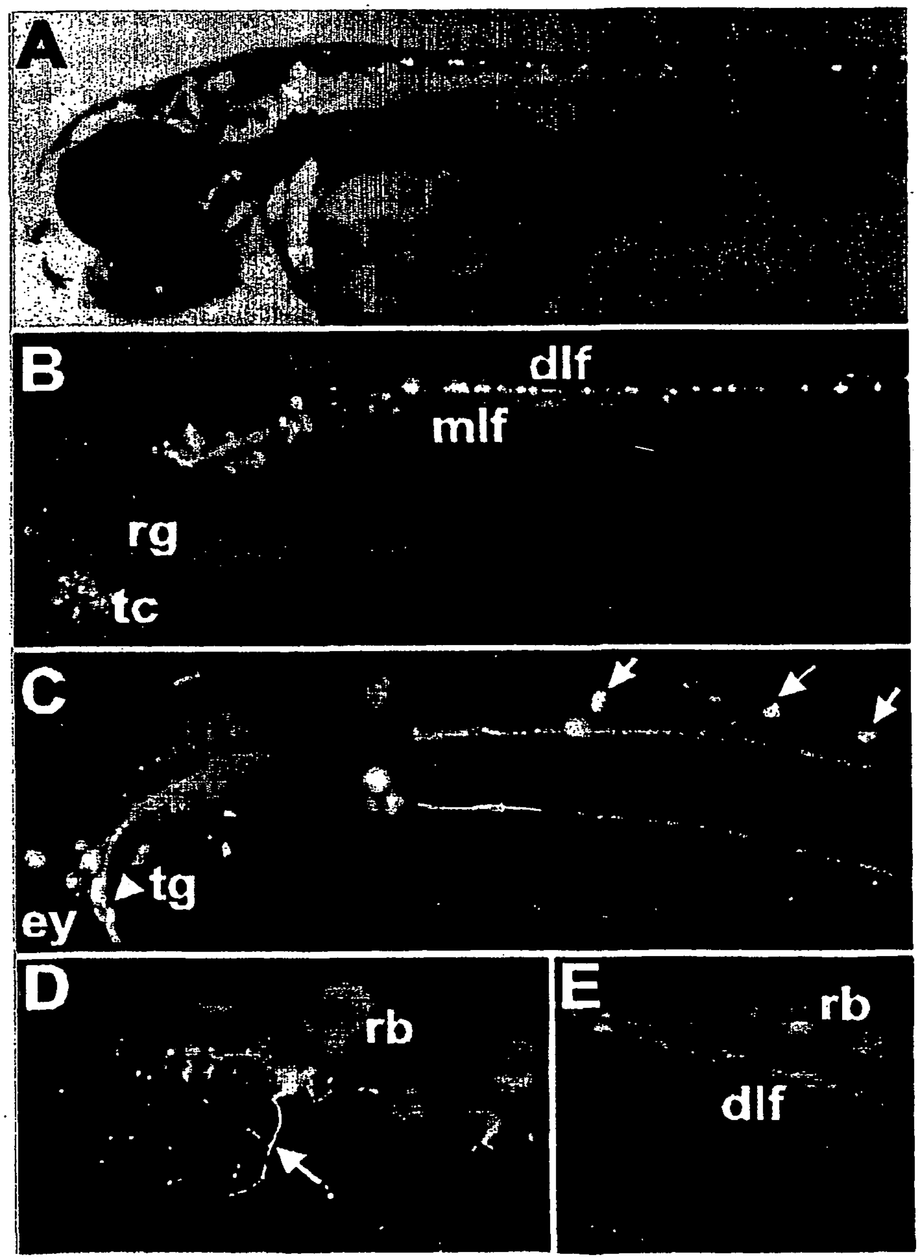

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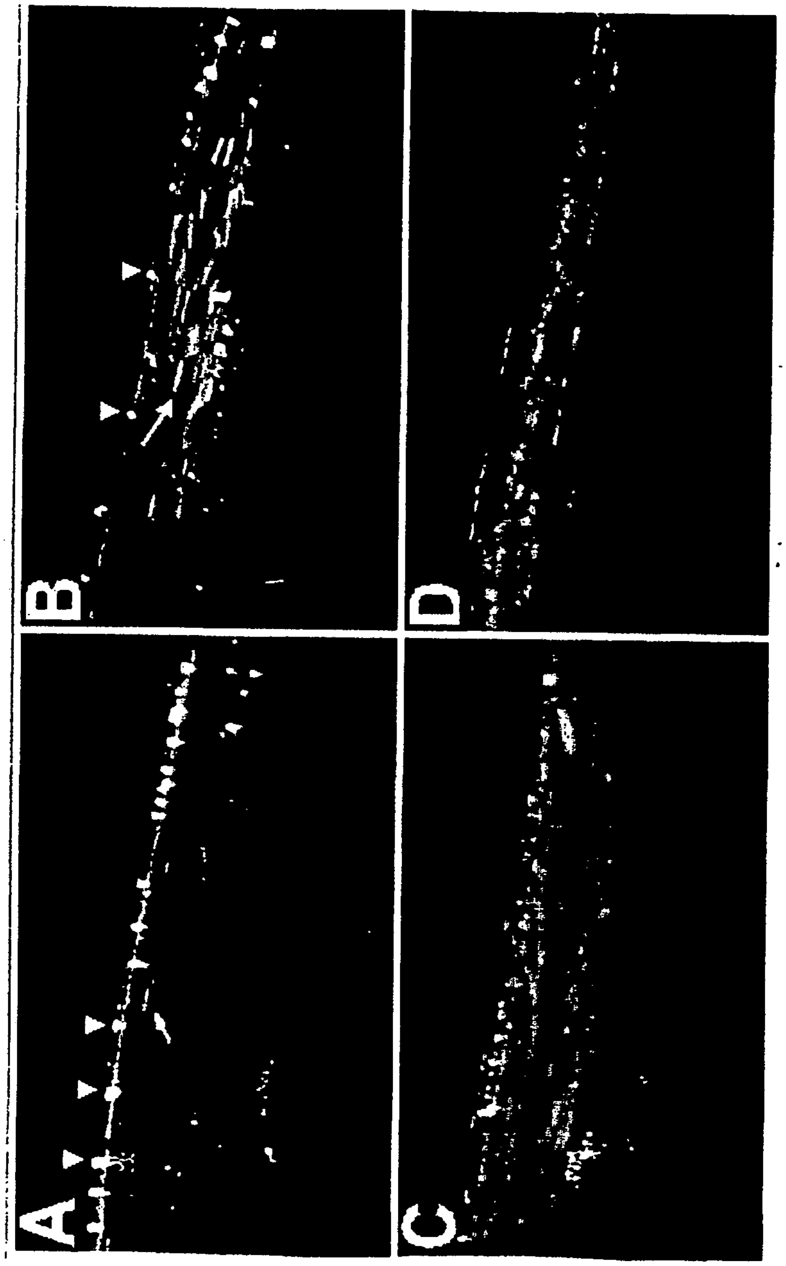
FIG. 6



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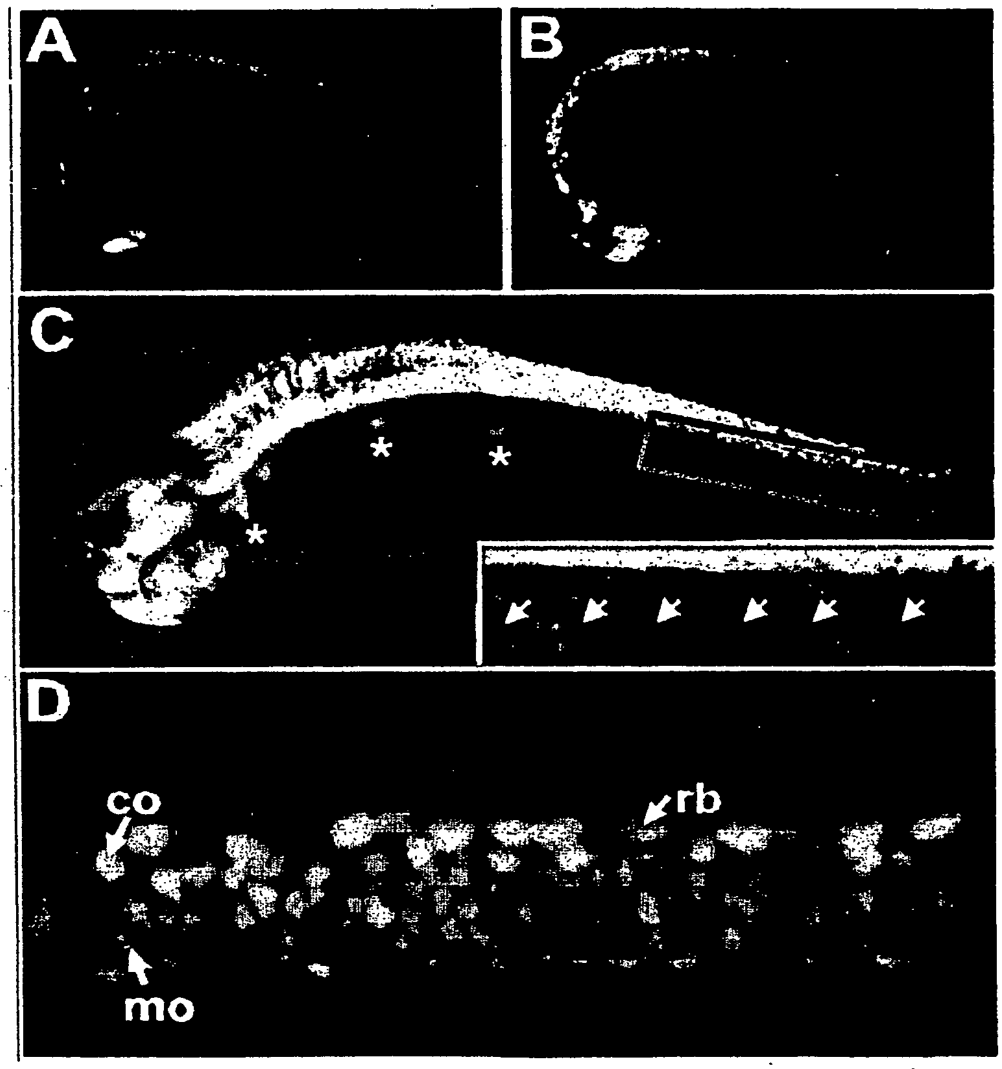

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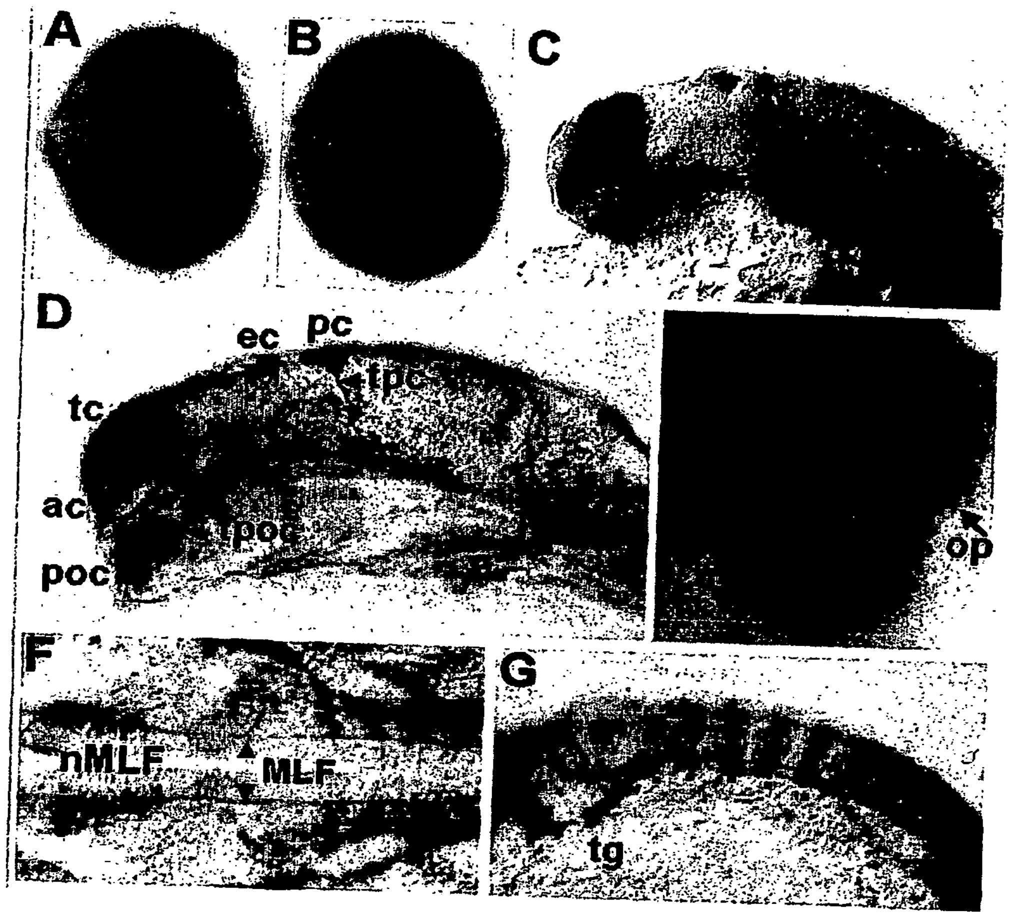
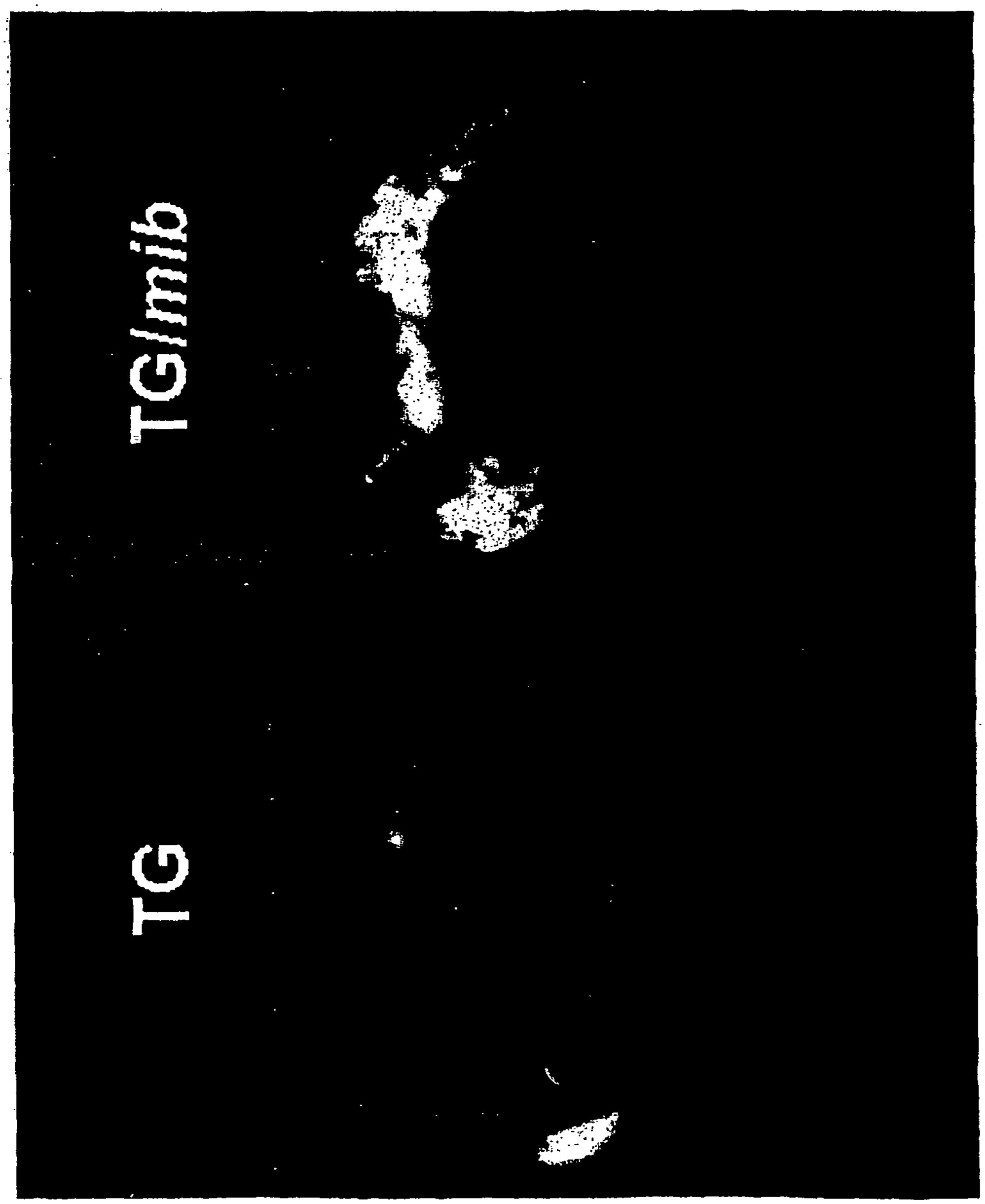


FIG. 9



SEQUENCE LISTINGS

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<120> ZEBRAFISH *HuC* PROMOTER CAPABLE OF DIRECTING NEURON-SPECIFIC EXPRESSION OF STRUCTURAL GENES, TRANSGENIC ANIMAL HAVING *HuC* PROMOTER AND ITS GENERATION, AND METHOD FOR SCREENING NEURONAL MUTANT ANIMALS USING THE TRANSGENIC ANIMAL

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/01259

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)
C12N 15/11, 15/12, 15/00, 15/62

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched
Korean Patents and Applications for Inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)
PubMed, Delphion, NCBI, PAJ, BIOPASS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Genbank Accession No. AF173984 (Park HC, Kim CH et al) 02 July 2000	1,2
P,X	PARK HC, KIM CH, BAE YK, YEO SY, HONG SK et al "Analysis of Upstream Elements in the HuC Promoter Leads to the establishment of transgenic zebrafish with fluorescent neurons" Dev Biol, vol. 227(15), p.279-293, Nov 2000 see the whole document	1-12
A	PARK HC, HONG SK, KIM HS, KIM SH, YOON EJ, KIM CH, MIKI N & HUH TL "Structural comparison of zebrafish ELav/Hu and their differential expression during neurogenesis" Neurosci Lett, vol. 279(2), p81-84, Jan 2000 see the abstract	9-12
A	AKAMATSU W, OKANO HJ, OSUMI N, INOUE T, NAKAMURA S, SAKAKIBARA SI, MIURA M, MATSUO N, DARNELL R & OKANO H "Mammalian ELAV-like neuronal RNA-bing protein HuB and HuC promote neuronal development in both the central and the peripheral nerve system" Neurobiology, vol. 96(17), p.9885-9890, 1999 see the whole document	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 NOVEMBER 2001 (16.11.2001)	19 NOVEMBER 2001 (19.11.2001)
Name and mailing address of the ISA/KR Korean Intellectual Property Office Facsimile No.	Authorized officer AHN, Mi-Chung Telephone No.